ATTACHMENT I

IMPROVED PRODUCTION OF CHYMOSIN IN *ASPERGILLUS* BY EXPRESSION AS A GLUCOAMYLASE-CHYMOSIN FUSION

Michael Ward, Lori J. Wilson, Katherine H. Kodama, Michael W. Rey, Randy M. Berka

Genencor Inc., 180 Kimball Way, South San Francisco, CA 94080.

We have extended the work on chymosin production in *Aspergillus* by constructing an expression vector in which the cDNA encoding bovine prochymosin B was fused in frame immediately following the codon for the last amino acid of the *A. awamori* glucoamylase (*glaA*) gene. Transformation of *A. awamori* with this plasmid led to the secretion of considerably higher amounts of chymosin than obtained with previous chymosin expression vectors. We present evidence that mature chymosin is autocatalytically released from the glucoamylase-chymosin fusion protein after secretion.

Bovine chymosin is an aspartyl protease extracted from the abomasum of unweaned calves and used as a milk clotting agent in cheese manufacture. The protein is initially synthesized as preprochymosin from which a 16 residue secretion signal is removed at secretion. At low pH the inactive prochymosin is processed to active chymosin by proteolytic removal of the propeptide[1]. There are two positions in the prosequence at which cleavage can occur and these two mechanisms can operate independently[2,3]. Processing at pH 2 occurs between residues 27 and 28 in the prosequence to yield active pseudochymosin and involves an intramolecular reaction between zymogen molecules. At pH 4.5 cleavage is autocatalytic and occurs after the 42nd residue of the prosequence to yield mature chymosin. Pseudochymosin is fairly stable at a pH below 3 or above 6 but is further processed to mature chymosin at pH 4.5.

Members of the genus *Aspergillus* have received considerable interest recently as the hosts for heterologous gene expression and foreign protein production[4–6]. The approach to this problem has generally been to utilize the promoter region from a highly expressed filamentous fungal gene and a secretion signal peptide fused to the coding sequence of the heterologous gene of choice. With one notable exception[6], this strategy has led to low levels (1–10 mg/l) of secreted product.

The *glaA* gene encodes glucoamylase and is highly expressed in many strains of *Aspergillus niger* and *Aspergillus niger* var. *awamori* (*A. awamori*). The gene has been cloned and an 18 amino acid secretion signal and a 6 residue propeptide were recognized[6,10]. In addition, it was deduced that the two forms of the secreted protein, differing in molecular weight, were the products of a single, differentially spliced gene. The promoter and secretion signal sequence of the *glaA* gene have been used in several attempts at heterologous gene expression in *Aspergilli*[4–6] including previous attempts to produce bovine chymosin in *Aspergillus nidulans* and *A. awamori*[4,11,12]. In the latter experiments a variety of constructs were made, incorporating prochymosin cDNA, either the glucoamylase or the chymosin secretion signal and, in one case, the first 11 codons of mature glucoamylase. Maximum yields of secreted chymosin obtained from *A. awamori* were below 15 mg/l in 50 ml shake flask cultures. In summary, these previous studies suggested that integrated plasmid copy number did not correlate with chymosin yields, abundant polyadenylated chymosin mRNA was produced, and intracellular levels of chymosin were high in some transformants regardless of the source of secretion signal. It was inferred that transcription was not a limiting factor in chymosin production but that secretion may have been inefficient. It was also evident that the addition of a small amino terminal segment of glucoamylase to the propeptide of prochymosin did not prevent activation to mature chymosin.

Here we have extended the work on chymosin production in *Aspergillus* by constructing an expression vector (pGAMpR) in which the cDNA encoding bovine prochymosin B was fused in frame immediately following the codon for the last amino acid of the *A. awamori glaA* gene. Transformation of *A. awamori* with this plasmid led to the secretion of considerably higher amounts of chymosin than obtained with previous chymosin expression vectors. We present evidence which suggests that mature chymosin is autocatalytically released from the glucoamylase-chymosin fusion protein after secretion.

Prochymosin cDNA has been expressed in other microbes, including *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. In several instances prochymosin was synthesized with an amino terminal extension consisting of a segment of a native protein from the host concerned. In *E. coli* prochymosin, with the first four amino acid residues replaced by an amino-terminal fragment of the *trpE* gene, has been produced under the control of the *trp* promoter[13]. The fusion protein accumulated as inclusion bodies in the cytoplasm but after appropriate extraction conditions it could be activated to yield mature chymosin. Moir et al.[14] described intracellular production of prochymosin in *S. cerevisiae*. The protein was synthesized with various segments of phosphoglycerate kinase, triosephosphate isomerase or galactokinase attached to the amino terminus, allowing increased production compared to direct expression from the same promoters. It was suggested that the increase in production was due to more efficient translation of the mRNA. Secretion of prochymosin, in the form of a fusion with the first few residues of invertase or alpha factor, was also obtained from *S. cerevisiae*[15]. The extracellular prochymosin was readily activated at low pH to give mature chymosin despite the additional amino acids on the prosequence. Similarly, activatable prochymosin was secreted from *Y. lipolytica* with either 14 or 90 residues of native alkaline extracellular protease attached to the amino terminus[15].

There are numerous examples of the production of heterologous proteins in *E. coli* as fusions with an entire native protein[16]. The rationale behind expression in this